NPY-Y7 RECEPTOR GENE

Field of Invention:

The present invention relates to isolated polynucleotide molecules which encode a novel neuropeptide Y (NPY) receptor designated NPY-Y7. In addition, the present invention relates to the use of these molecules in the production of NPY-Y7 receptors using recombinant DNA technology and to methods of screening and testing compounds for agonist or antagonist activity.

Background of the Invention:

Neuropeptide Y (NPY) forms a family (called the pancreatic polypeptide family) together with pancreatic polypeptide (PP) and peptide YY (PYY), which all consist of 36 amino acids and possess a common tertiary structure. NPY receptors, members of the G protein- coupled receptor superfamily, when activated influence a diverse range of important physiological parameters, including effects on psychomotor activity, central endocrine secretion, anxiety, reproduction, vasoactive effects on the cardiovascular system and strongly stimulates food consumption. Specific agonists and antagonists of NPY are therefore likely to be of substantial benefit for therapy of a wide range of clinical disorders. As NPY possess a compact tertiary structure and different parts of the molecule are required for interaction with different subtypes of the receptor, the logical developments of both agonists and antagonists is critically dependent upon the availability and knowledge of specific receptor structure.

It is presently known that NPY binds specifically to at least six receptors; Y1, Y2, Y3, Y4, Y5 (or "atypical Y1") and Y6. While it has been demonstrated that NPY receptors couple to the adenylate cyclase second messenger system, it remains probable that additional NPY receptor subtypes exist since there is evidence that phosphatidylinositol turnover, cations, and arachidonic acid may also function as second messengers for NPY.

Since NPY agonists and antagonists may have commercial value as, for example, potential anti-hypertensive agents, cardiovascular drugs, neuronal growth factors, anti-psychotics, anti-obesity and anti-diabetic agents, the ability to produce NPY receptors by recombinant DNA technology would be

advantageous. To this end, DNA molecules encoding Y1, Y2, Y4, Y5 and Y6 have previously been isolated.

The present inventors have now isolated novel DNA molecules encoding the human and murine NPY-Y7 receptors.

Summary of the Invention:

Thus, in a first aspect, the present invention provides an isolated polynucleotide molecule encoding an NPY-Y7 receptor or a functionally equivalent fragment thereof.

The encoded NPY-Y7 receptor is characterised by the N-terminal amino acid sequence:

$MX_1X_2MX_3EKWDX_4NSSE$ (SEQ ID NO: 1),

wherein X_1, X_2, X_3 and X_4 are selected from codable amino acids but, preferably, X_1 is selected from Phe and Ser, X_2 is selected from Ile and Thr, X_3 is selected from Asn and Ser, and X_4 is selected from Thr and Ser.

More preferably, the polynucleotide molecule encodes a human NPY-Y7 receptor of about 408 amino acids or a murine NPY-Y7 receptor of about 405 amino acids.

Most preferably, the polynucleotide molecule encodes a human NPY-Y7 receptor having an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 2 or a murine NPY-Y7 receptor having an amino acid sequence subtantially corresponding to that shown as SEQ ID NO: 3.

The polynucleotide molecule may comprise a nucleotide sequence substantially corresponding or, at least, showing at least 90% (more preferably, at least 95%) homology to that shown at nucleotides 1 to 1903 or nucleotides 369 to 1592 of SEQ ID NO: 4 or any portion thereof encoding a functionally equivalent NPY-Y7 receptor fragment.

The polynucleotide molecule may be incorporated into plasmids or expression vectors (including viral vectors), which may then be introduced into suitable bacterial, yeast, insect and mammalian host cells. Such host cells may be used to express the NPY-Y7 receptor.

Accordingly, in a second aspect, the present invention provides a mammalian, insect, yeast or bacterial host cell transformed with the polynucleotide molecule of the first aspect.

In a third aspect, the present invention provides a method of producing NPY-Y7 receptors or functionally equivalent fragments thereof, comprising

culturing the host cell of the second aspect under conditions enabling the expression of NPY-Y7 receptors or functionally equivalent fragments thereof.

Preferably, the host cell is mammalian or of insect origin. Where the cell is mammalian, it is presently preferred that it be a Chinese hamster ovary (CHO) cell, monkey kidney (COS) cell or human embryonic kidney 293 cell. Where the cell is of insect origin, it is presently preferred that it be an insect Sf9 cell.

In a preferred embodiment, the NPY-Y7 receptors or functionally equivalent fragments thereof are expressed onto the surface of the host cell.

The polynucleotide molecule of the present invention encodes an NPY receptor which may be of interest both clinically and commercially as it is expressed in many regions of the body and neuropeptides of the NPY family affect a wide number of systems.

By using the polynucleotide molecule of the present invention it is possible to obtain NPY-Y7 receptor protein or fragments thereof in a substantially pure form.

Accordingly, in a fourth aspect, the present invention provides a NPY-Y7 receptor or a functionally equivalent fragment of said receptor, in a substantially pure form.

In a fifth aspect, the present invention provides an antibody or fragment thereof capable of specifically binding to the NPY-Y7 receptor or functionally equivalent fragment of the fourth aspect.

In a sixth aspect, the present invention provides a non-human animal transformed with the polynucleotide molecule of the first aspect of the present invention.

In a seventh aspect, the present invention provides a method for detecting agonist or antagonist agents of an NPY-Y7 receptor, comprising contacting an NPY-Y7 receptor, functionally equivalent fragment thereof or a cell transfected with and expressing the polynucleotide molecule of the first aspect, with a test agent under conditions enabling the activation of an NPY-Y7 receptor, and detecting an increase or decrease in activity of the NPY-Y7 receptor or functionally equivalent fragment thereof.

An increase or decrease in activity of the receptor or functionally equivalent fragment thereof may be detected by measuring changes in cAMP production, Ca^{2+} levels or IP3 turnover after activating the receptor or fragment with specific agonist or antagonist agents.

In a further aspect, the present invention provides an oligonucleotide or polynucleotide probe comprising a nucleotide sequence of 10 or more nucleotides, the probe comprising a nucleotide sequence such that the probe specifically hybridises to the polynucleotide molecule of the first aspect under high stringency conditions (Sambrook et al., *Molecular Cloning: a laboratory manual*, Second Edition, Cold Spring Harbor Laboratory Press).

In a still further aspect, the present invention provides an antisense oligonucleotide or polynucleotide molecule comprising a nucleotide sequence capable of specifically hybridising to an mRNA molecule which encodes an NPY-Y7 receptor so as to prevent translation of the mRNA molecule.

Such antisense oligonucleotide or polynucleotide molecules may include a ribozyme region to catalytically inactivate mRNA to which it is hybridised.

The polynucleotide molecule of the first aspect of the invention may be a dominant negative mutant which encodes a gene product causing an altered phenotype by, for example, reducing or eliminating the activity of endogenous NPY-Y7 receptors.

The term "substantially corresponding" as used herein in relation to amino acid sequences is intended to encompass minor variations in the amino acid sequences which do not result in a decrease in biological activity of the NPY-Y7 receptor. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkalamino acids.

The term "substantially corresponding" as used herein in relation to nucleotide sequences is intended to encompass minor variations in the nucleotide sequences which due to degeneracy in the DNA code do not result in a change in the encoded protein. Further, this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system but in which the variations do not result in a decrease in biological activity of the encoded protein.

The term "functionally equivalent fragment/s" as used herein is intended to refer to fragments of the NPY-Y7 receptor that exhibit binding specificity and activity that is substantially equivalent to the NPY-Y7 receptor from which it/they is/are derived.

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

Reference to percent homology made in this specification have been calculated using the BLAST program blastn as described by Altschul, S.F. et al., "Capped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Research*, Vol. 25, No. 17, pp. 3389-3402 (1997).

Brief description of the accompanying Figures:

Figure 1 shows the degree of identity between the predicted amino acid sequence of the human NPY-Y1, NPY-Y2 and NPY-Y7 receptors.

Figure 2 provides a graph showing the inhibition of human [^{125}I]PYY binding with various NPY-related peptides on human NPY-Y7 membranes. The results were obtained through competitive displacement of [^{125}I]PYY on membranes of COSm6 cells transiently expressing human NPY-Y7 receptors. Membranes were incubated with [^{125}I]PYY (50pM) and increasing concentrations of peptide competitors. Data are representative of a single experiment with each point measured in triplicate.

Figure 3 provides a schematic diagram of the murine NPY-Y7 receptor gene. The gene covers approximately 12 kb and consists of three exons.

Figure 4 shows the degree of identity between the predicted amino acid sequence of the human and murine NPY-Y7 receptors.

Detailed Disclosure of the Invention:

Human NPY-Y7 cDNA

Human amygdala and testis cDNA libraries (Stratagene) were screened under low strigency conditions with a 401 bp ^{32}P-labelled fragment (corresponding to nucleotides 507 to 908 of SEQ ID NO: 4) originated from a human fetal brain EST clone (GenBank AA449919). Two overlapping cDNA clones were obtained from the screen. The combined nucleotide sequence (hy7) of the clones is shown as SEQ ID NO: 4 and encodes a protein of 408 amino acids (SEQ ID NO: 2).

Sequence comparison with other G protein coupled receptors identified neuropeptide Y receptors as the most closely related group with approximately 32% amino acid sequence identity to the Y1 receptor subtype (Figure 1). Further, *in situ* hybridisation studies of rat brain sections has identified a NPY-Y7 mRNA distribution (expression was found to occur in the amygdala, the CA3 region of the hippocampus and the piriform cortex) which is consistent with the expression of other NPY-receptor subtypes (Blomquist, A.G., and Herzog, H., TINS 20(7), 1997) and is in agreement with the suggestions of the existence of further Y-receptor family members. This mRNA distribution suggests important functions for the NPY-Y7 receptor in the regulation of the circadian rhythm, anxiety and metabolic status.

Radio-ligand binding experiments has shown that the protein encoded by the hy7 cDNA shows highest affinity for human PYY (Figure 2). These experiments were conducted using COS-6 or HEK (293) cells transiently expressing recombinant Y7 receptor protein. The radio-ligand binding (Herzog, H. et al., Proc. Natl. Acad. Sci. USA 89:5794-5798, 1992) suggests that the NPY-Y7 receptor has a pharmacology similar to the Y2 receptor (Rose, P., J. Biol. Chem. 270:22661-22664, 1995). The rank of potency for the Y7 receptor is:

PYY>NPY>[2-36]PYY>[3-36]NPY>[13-36]NPY>>(Leu31, Pro34)NPY>PP.

Chromosomal Localisation of the Human Y7 gene

Screening of a medium resolution Stanford G3 panel of 83 clones was performed to further refine the map position of the hy7 gene. PCR amplification was carried out on this panel using primers hy7-A (5'GGATGGCCATTTGGAAAC3') and hy7-B (5'CCAATCCTTCCATACATG3'), corresponding to nucleotides 507-524 and 890-907 of the hy7 cDNA (SEQ ID NO: 4), respectively. The analysis indicated that the hy7 gene is most closely associated with the marker SHGC-418 on the long arm of chromosome 4. This map location is defined by markers AFM191xh2 and AFM347ZH1. Assessment of the flanking markers using the Whitehead/MIT STS-Based ~~Map of the Human Genome)(http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map) in conjunction with The Genome Directory (Adams~~, M.D., et al. Nature 377 Suppl. (1995)) identifies 4q21.3 as the most likely position of the hy7 gene.

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Mouse Y7 genomic DNA

Using a ^{32}P-labelled fragment of the hy7 cDNA a mouse genomic BAC library (Genome Systems) was screened. A clone encoding the entire gene of the mouse equivalent to hy7 was isolated (SEQ ID NO: 5). The gene covers approximately 12 kb and is divided by two introns into three exons (Figure 3). Figure 4 shows the degree of identity between the predicted amino acid sequence of the human and murine NPY-Y7 receptors.

Pharmacological characterisation

pcDNA3.1-hy7 cDNA was transiently transfected into the COSm6 cell line using FUGENE and 5mg of DNA/106 cells. The COSm6 cells were grown in Dulbecco's modified Eagles medium supplemented with 2mM glutamine and 10% fetal calf serum, in 5% CO_2 at 37°C. Membranes were harvested with COSm6 cells 72hr post-transfection. Adherent cells were washed twice in ice-cold phosphate buffered saline and lysed using a glass homogeniser in ice-cold hypotonic buffer (50mM Tris-HCI, pH 7.4, 0.1% bacitracin). Membranes were pelleted by high speed centrifugation (30,000 x g, 15min, 4°C), homogenised again in ice-cold hypotonic buffer and collected again by high speed centrifugation (30,000 x g, 15min, 4°C). The final membrane pellet was resuspended into 1ml of ice-cold binding buffer (50mM Tris-HCI, pH7.4, 10mM NaCl, 5mM MgCl2, 2.5mM CaCl2, 0.1% bacitracin, 0.1% bovine serum albumin. Membrane suspensions were diluted in binding buffer to yield membrane protein concentrations of 0.05mg/ml. Under these conditions non-specific binding of [^{125}I]PYY to membranes was less than 10%. [^{125}I]PYY and unlabelled peptide competitors were also diluted to the required concentrations in binding buffer. Samples were prepared by mixing 50ml binding buffer, unlabelled peptide or binding buffer (50ml), [^{125}I]PYY (50mM, 50ml) and membrane suspension (100ml). Samples were incubated at room temperature for 2hr. Incubations were terminated by centrifugation (4min) and pellets collected. Radioactivity was measured for 1min in a g counter.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.